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To:	<u>Examiner Lilling</u>		From:	Irwin M. Aisenberg <i>(Carol Duff)</i>	
Fax:	703-746-5121		Phone:		
Date:	November 24, 2003		Pages:	7	(including cover page)
Re:	U. S. Patent Application No. 10/056,063		Cc:		
Our Ref. No.:	P66143US1	Your Ref. No.:			
<input type="checkbox"/> Important <input type="checkbox"/> For Submission <input type="checkbox"/> Please Comment <input type="checkbox"/> Please Reply <input checked="" type="checkbox"/> As Requested					

Dear Examiner Lilling:

Further to our telephone conversation today, please find attached a copy of the Declaration executed by M. Clark Dale dated November 5, 2003.

Cordially yours,

 Carolina M. Duff
 Secretary to Irwin M. Aisenberg

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of:

M. Clark DALE *et al.*

Attorney Dkt. No.: P66143US1

Application No.: 10/056,063

Group Art Unit: 1651

Filed: January 28, 2002

Examiner: Herbert J. Lilling

For: A HIGH SPEED, CONSECUTIVE BATCH OR CONTINUOUS, LOW EFFLUENT
PROCESS FOR THE PRODUCTION OF ETHANOL FROM MOLASSES,
STARCHES OR SUGARS

DECLARATION

I, M. Clark Dale, am one of the inventors of the above-identified application.

The accompanying material (10 pages) entitled "Side-by-side Comparison of Strain *Saccharomyces cerevisiae* (Sc) BPSC-15 (NRRL 30630) with a standard yeast strain Sc CBS 295S" reflects work done by me or under my direct supervision.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statement may jeopardize the validity of the application or any patent issued thereon.

Signed this 5th day of November 2003


M. Clark Dale

**Side-by-side comparison of strain
Saccharomyces cerevisiae (Sc) BPSC-15 (NRRL 30630)
with a standard brewing yeast strain Sc CBS 2959.**

Introduction

The performance the *Saccharomyces cerevisiae (Sc)* strain BPSC 15 developed by Dale & Moelhman was compared on an identical side by side basis with a top performing Sc brewing strain suggested by Asturias (USP 4,560,659) following the suggestion of USP Examiner Lilling. Strain Sc CBS 2959 is suggested by Asterias as being a superior strain for ethanol production from cane sugar (Example 1, Example 2). This strain was ordered and obtained from the Centraalbureau von Schimmelcultures, Netherlands.

Example 5. A side-by-side performance comparison of Sc BPSC-15 with a conventional brewing strain of Sc (CBS- 2959).

Strains Sc BPSC-15 and Sc CBS-2959 were each grown-up from 1 ml inoculums in 100 ml of sterile YMP growth media A (10 g/L glucose, 3 g/L each of yeast extract, malt extract, and peptone) in twin 250 ml Erlenmyer flasks fitted with a magnetic stirring bar and a foam plug which was protected with aluminum foil. The cultures were grown for 24 hours with gentle magnetic stirring (100 RPM) at 28°C

50 ml of this growth medium was poured off, and 150 ml of sterile 21% sucrose fermentation medium B (210 g/L sucrose, 3 g/L each of yeast extract, malt extract, and peptone nutrients) was added to the residual 50 ml of yeast culture bringing the total volume to 200 ml. The fermentation flasks were stirred at 100 RPM using the magnetic stir bars in an incubator held at 28 to 30°C.

Sterile samples of the two fermentations were taken to monitor the fermentations. Brix of the media was monitored by refractive index, free cell density monitored by absorbance at 660 nm using a Perkin Elmer spectrophotometer, and compositions of the fermentation broth determined by HPLC analysis. When the fermentations were near completion, 160 ml of the media was poured off, and a second- consecutive batch- fermentation started in the same flask in a manner suggested both by Asturias (Example 2) and by Dale & Moelhman.

Data from the two strains' consecutive fermentations are presented in Table 1 and brix of the fermentations over 1 week trials are shown in Figure 1. The initial fermentations were very comparable, with a brix of 6 reached

by both in about 40 hours from a starting brix of 15. However, striking differences in free cell density were immediately obvious. Free cell density of the CBS 2959 was measured at 2.5 g/L at 17 hours into the first fermentation and increased to 5.7 g/L at 40.5 hours (near completion of the fermentation), the BPSC 15 strain showed a free cell density of only 0.3 g/L at 10 hours, and was still only 0.32 g/L at 40 hours. Nearly all of the BPSC yeast were in small 1-3 mm diameter flocc pellets on the bottom of the flask. A second consecutive batch fermentation of both strains was started at hour 40.5 of the trials, with 160 ml of the fermentation broth poured off (40 ml allowed to remain in the flask), and the volume brought back to 200 ml with the 21 brix fermentation media B. During consecutive batch #2, BPSC-15 began to significantly outperform the CBS 2959, with the BPSC-15 achieving a brix of 9 (89% completion/sugar utilization) in 24.5 hours, while the CBS was still at a brix of 12.8 (only 52% completion). Both fermentations were reset with the same procedures as before (pour off 160 ml, replace w/ fresh medium B), and fermentation #3 started. In 23.5 hours, the BPSC-15 fermentation was nearly complete (8.2 brix, 97% completion/sugar utilization) while the CBS 2959 was only 28% complete. We reset the BPSC 15 flask, but allowed the CBS 2595 to continue fermentation #3. The BPSC 15 flask continued to complete fermentations #4, #5, and #6 in 11 to 15 hours, while the CBS required 62.5 hours to achieve 87% sugar utilization in fermentation #3. Both flasks were reset (ferm. #4 for CBS 2959, #7 for BPSC 15) at 133 hours. Fermentation #7 for the BPSC 15 was 97% complete in 13.8 hours, while the CBS 2959 was only 41% complete.

Based on identical medium(s), handling/inoculation/reset procedures, the BPSC 15 yeast was able to ferment the 210 g/L sucrose media solution at an average rate of 11.1 g sucrose /L h, based on data from fermentations #2 through #7, while the CBS 2959 showed an average fermentation rate of 3.9 g sucrose /L h for fermentations # 2 through #4. If the BPSC 15 yeast settled volume were allowed to continue to build to 30% v/v (60 ml settled volume in a 200 ml fermentation) the BPSC-15 would be completing consecutive batch fermentations of 21 brix sucrose feeds in 8 hours or less as described in previous examples (25 g sucrose/ L h utilization). This compares to the best performance from the CBS 2959 strain of 4.6 g sucrose/L h maximal rate or complete fermentation of 210 g/L sucrose in 45 hours.

This High Speed (HS) fermentation of the BPI strain is due to the highly stable flocculent nature developed in the BPSC 15 strain. The free cell density in the BPSC-15 flask ranged from 0.04 to 0.24 g/L, so low as to

leave an almost crystal clear media as shown in Photos 1 & 2 (taken at hour 6 of fermentation #2 for both flasks) and in Photos #3 &4 (taken at hour 133 (hour 62 of fermentation #3 for CBS 2959, hour 10.45 of fermentation #6 for BPSC-15). The CBS-2959 strain, in contrast behaves as all standard brewing strains of *Saccharomyces cerevisiae*, with a free cell density increasing to some maximal value (approximately 4.4-5.5 g/L) and eventually showing some flocculent characteristics once the fermentation is completed. The free cell density of the CBS 2959 strain ranged from 0.9 to 5.5 g/L, while the average free cell density of the BPSC 15 strain was only 0.11 g/L, a factor of 30 X less than the CBS 2959 average density. The volume of floc pellets in the BPSC 15 flask approached 40 ml (instantaneous settling volume) by fermentation # 5.

The ability to quickly and routinely complete a fermentation in 8 hours using strain BPSC-15 versus the industry standard of 50 to 70 hours with standard brewing strains, allows fermenter size to be reduced by a factor of about 5 X, translating to large capital savings in new facilities, or to allow current ethanol facilities to expand output by up to 5X using current fermenters. There are further advantages associated with the new strain such as eliminating the need for cleaning chemicals between batches, reduced labor, the production of a valuable side product- yeast paste, a higher efficiency conversion of sugars to ethanol, etc.

Photo #1- Fermentation Flasks of Comparison Trials between BPSC-15 and CBS 2959 – Hour 6 of Fermentation #2 w/ flash

Photo #2- Fermentation Flasks of Comparison Trials between BPSC-15 and CBS 2959 – Hour 6 of Fermentation #2 -side lit

Photo #3- Fermentation Flasks of Comparison Trials between BPSC-15 (Hour10.5 Fermentation #6) and CBS 2959 (Hour 62 of Fermentation #3) w/ flash

Photo #4- Fermentation Flasks of Comparison Trials between BPSC-15 (Hour10.5 Fermentation #6) and CBS 2959 (Hour 62 of Fermentation #3) – side lit

Table 1. Direct Comparison between Sc strains BPSC 15 and CBS 2959

Date	Time	CBS 2959										BPSC-15									
		Total Hrs	Ferm Hours	Ferm #	CBS Brix	% Comp	CBS Abs(OD)	Free Yst Dens.	Ferm Hours	BPSC Ferm #	BPSC Brix	BPSC % Compl	Ferm ABS(OD)	Free Yst Dens.							
25-Oct	18:30	0.0	0	1	15.1	0%	n.m.		0	1	15.1	0%	n.m.								
26-Oct	11:30	17.0	17	1	11	44%	9.9	2.4	17	1	13.8	14%	0.74	0.18							
27-Nov	11:00	40.5	40.5	1	6	97%	23.1	5.5	40.5	1	6	97%	0.85	0.20							
27-Oct	11:00	40.6	0	2	18.1	0%	4.62	1.1	0	2	18.1	0%	0.17	0.04	Photo #1 & 2						
27-Oct	17:00	46.5	6	2	17.2	9%	10.54	2.5	6	2	17	11%	0.46	0.11							
28-Oct	11:30	71.0	24.5	2	12.8	52%	17.9	4.3	24.5	2	9	89%	1	0.24							
28-Oct	11:30	71.1	0	3	19.8	0%	3.58	0.9	0	3	18.6	0%	0.2	0.05							
28-Oct	20:00	79.5	8.5	3	19.5	3%	11.5	2.8	8.5	3	15.4	30%	0.3	0.07							
29-Oct	10:30	94.5	23.5	3	16.5	28%	18.4	4.4	23.5	3	8.2	97%	0.8	0.19							
29-Oct	10:30	94.6							0	4	19	0%	0.16	0.04							
29-Oct	21:30	105.5	34.5	3	14.5	45%	n.m.		11	4	10.9	73%	n.m.								
29-Oct	23:15	107.3							12.75	4	9.9	82%	n.m.								
29-Oct	107.3								0	5	19.2	0%									
30-Oct	14:45	122.8	51.75	3	11.5	70%	n.m.		15.5	5	11.4	69%									
30-Oct	15:00	123.0	3						0	6	19.2	0%									
31-Oct	1:10	133.2	62.1	3	9.4	87%	19.5	4.7	10.45	6	11.4	69%									
31-Oct	1:45	133.7	0	4	19	0%	3.9	0.9	0	7	19.3	0%									
31-Oct	9:15	143.0	9.25	4	16.8	20%	n.m.		9.25	7	13	55%									
31-Oct	15:30	147.6	13.8	4	14.5	41%	n.m.		13.8	7	8.3	97%	0.18	0.04							
1-Nov	9:30	167.3	33.5	4	11	72%	16.3	3.4													

Photo #3 & 4

Yeast Performance Comparison

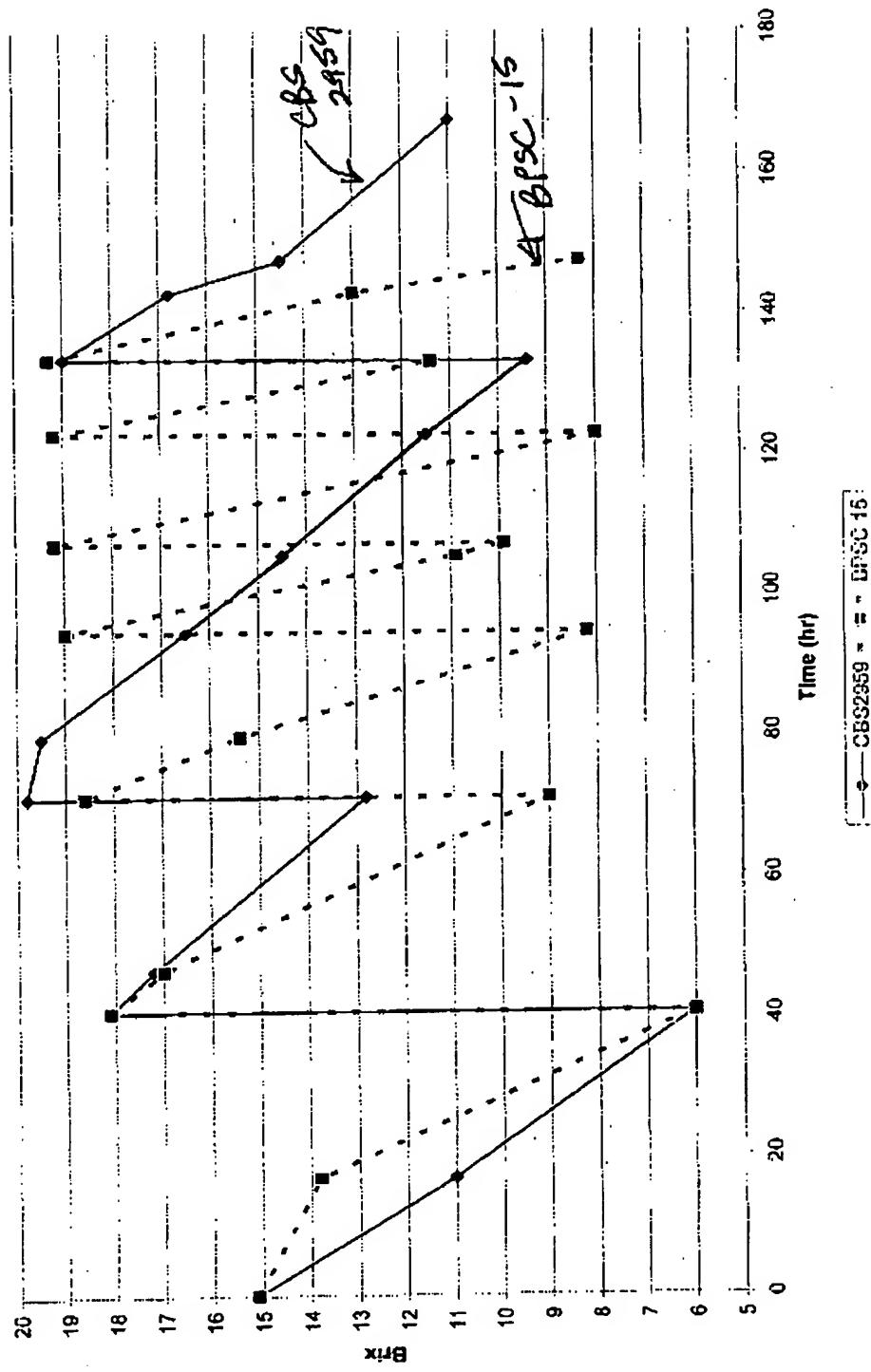


Figure 1. Brix vs. time for Consecutive Batch Fermentation Comparison of strains CBS-15 and CBS 2959